

## Composition Studies on Tobacco. XVI. Steam-Volatile Acids

By IRWIN SCHMELTZ, R. L. STEDMAN, and R. L. MILLER (Eastern Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Philadelphia 18, Pa.)

The lower fatty acids of various types and grades of tobacco were studied by gas chromatography and identified by chromatographic and infrared spectral characteristics. Acids from  $C_1$  or  $C_2$  to  $C_9$  were found, including four ( $n$ - $C_4$ ,  $n$ - $C_7$ ,  $n$ - $C_8$ , and  $n$ - $C_9$ ) not previously reported in tobacco leaf. Among the tobaccos investigated were bright (aromatic and aroma-deficient grades), burley, Maryland, and Turkish (Smyrna and Samsun). In general, all samples were qualitatively similar in volatile acid composition. Quantitatively the tobacco types differed as follows: Turkish (Samsun) contained a much higher level of  $\beta$ -methylvaleric acid (greater than four times) and a lower level of combined acetic and formic acids (less than one-half) than bright, and had a much higher proportion of isovaleric to  $n$ -valeric acid. Burley and Maryland contained lower levels of all identified acids than Turkish (Samsun) and bright. Among the grades of bright, the aromatic tobaccos contained more volatile acidic material than the aroma-deficient tobaccos, and showed other differences in distributions of lower fatty acids compared to aroma-deficient samples.

The aroma of cigarette tobacco and its smoke is presently of considerable interest, and studies on this subject have been underway here for some time. One objective of this work is to determine the aromatic contributions of the four tobacco types in most

domestic cigarettes, i.e., bright, burley, Maryland, and Turkish tobaccos, to over-all cigarette aroma. Another objective is to determine why tobaccos of the same type may differ in aroma. As part of this work, a study of the volatile, neutral fraction of tobaccos of different type or aromatic properties was recently completed (1). Fourteen neutral components were quantitatively evaluated and tentative identifications were made for furfural, furfuryl alcohol,  $n$ -capraldehyde, neophytadiene,  $m$ -tolualdehyde, and benzyl acetate. Also, some quantitative differences in samples having different aromatic properties were observed. Similarly, a study of blended and unblended cigarettes was conducted (2). At least 66 neutral components were observed and data were obtained on the identification of five of these. The present report is an extension of this work and concerns the volatile acids of leaf.

The presence of certain common volatile acids in tobacco and smoke is well known. Onishi, *et al.* (3-6) have studied the volatile acids of flue-cured tobacco by classical methods and have found differences between American and Japanese tobaccos. Buyske, *et al.* (7) and Izawa and Kobashi (8) have investigated the volatile acids of cigarette smoke. Two recent reviews have listed the known volatile acids found in leaf and smoke (9, 10). However, the volatile acid fraction of various tobacco types or tobaccos of different aromaticities have not been studied by gas chromatographic methods. Since subtle variations in chemical composition frequently result in gross organoleptic differences, the use of gas chromatography appeared essen-

tial in this problem, and the results of a preliminary study (11) confirmed the feasibility of such an approach.

### METHOD

The tobaccos, method of steam distillation, and certain phases of the fractionation have been previously described (1). In general, 100 g of tobacco was steam distilled and the distillate was extracted with ether after saturation of the distillate with NaCl. Bases were removed from the ether extract with aqueous HCl and then the acids were removed from the ether by extraction with 5% NaOH solution (total volume, 135 ml). The alkaline solution was then acidified to pH 1.0 with HCl and saturated with NaCl. The acidified extract was extracted with redistilled ether 5 times (total, 250 ml) and, after separation of layers, the ether solution was washed with successive 5 ml portions of NaCl-saturated water until the washings were neutral to phenolphthalein. The washed ether solution was then dried over magnesium sulfate and reduced in volume to 1.0 ml (concentrate A) on the Stedman and micro spinning band columns as previously described (12).

Concentrate A was investigated by gas chromatography using<sup>1</sup> Tween 80-H<sub>3</sub>PO<sub>4</sub> or diethylene glycol adipate polyester (DEGA)-H<sub>3</sub>PO<sub>4</sub> columns in either an F & M Model 500 instrument with thermal conductivity detection or a Perkin-Elmer Model 800 dual column, flame ionization chromatograph. The F & M instrument was operated either isothermally at 110°C (2' × 0.25" column of 25% Tween 80 and 2% H<sub>3</sub>PO<sub>4</sub> on 60-80 mesh Gas Chrom P) or programmed (4° per min. from 100 to 211°C, 2' × 0.25" column of 25% DEGA and 2% H<sub>3</sub>PO<sub>4</sub> on 60-80 mesh Gas Chrom P) with a helium flow rate of 60 ml per min. and injector and detector temperatures of 260 and 290°C, respectively. The Model 800 was operated isothermally at 110°C (5' × 0.13" Tween column of above composition) or at 125°C (6' × 0.13" column of 25% DEGA-2% phosphoric acid on silanized 80-100 mesh Chromosorb W) with an injector temperature of 170°C, a helium flow rate of 40 ml per min., and hydrogen and air pressures of 20 lb and 38 lb, respectively; the detector and column temperatures were the same.

Quantitative comparisons were made on the Perkin-Elmer instrument. Usually, injections

of 1-5  $\mu$ l concentrate A yielded optimal peak sizes for measurements in the range required for linearity of detector response with concentration. Peak area was measured with a planimeter and for each peak an "equivalent peak area" (EPA) was calculated (5) by  $EPA = (10^5 A/VW)$ , in which  $A$  was the measured peak area (cm<sup>2</sup>),  $V$  was the volume ( $\mu$ l) of the injected aliquot, and  $W$  was the calculated weight (g) of moisture-free tobacco used in the distillation. In general, EPA represents a theoretical peak area which would be obtained on single injection of the entire amount of steam-volatile acidic substances from 100 g (moisture-free basis) of tobacco. Limitations on quantitative comparisons by EPA measurements have been previously discussed (1) and further details are given below.

Several paper chromatographic methods (13-16) were used in identification studies on concentrate A and on the gas chromatographic fractions obtained by resolution of the concentrate on the Tween column in the Model 500 instrument.

Large batches (10 lb) of tobacco had to be steam distilled to obtain enough material for identification by both paper chromatography and infrared spectrophotometry. The chromatograms from the large batches were essentially similar to those obtained with the 100 g quantities of tobacco used in the quantitative comparisons.

It should be noted that the method measures the acids which steam distill from the tobacco at its natural pH in contrast to common analytical techniques (17, 18) which measure total (free and combined) volatile acids without resolution of components.

### Results and Discussion

Comparative studies on the Tween and DEGA columns indicated significant differences in resolution of authentic volatile fatty acids. Formic was eluted after acetic and the two acids were resolved on the Tween-phosphoric acid column, although resolution of formic and propionic was difficult when either acid was in large excess. Formic and acetic were eluted together and before propionic on the DEGA-phosphoric acid column. Isocaproic and  $\beta$ -methylvaleric acids were partially resolved on both columns, depending on the relative amounts present. For  $n$ - and iso-acids from C<sub>3</sub> to C<sub>8</sub>, the iso-acid was eluted before the parent  $n$ -acid on both columns.

<sup>1</sup> Mention of a special commercial product does not constitute endorsement by the U.S. Department of Agriculture over similar products.

Losses of acids due to volatilization during solvent (ether) removal were studied in a manner similar to that reported earlier for the methyl esters of such acids (12). Although gross losses of the methyl esters occurred on ether evaporation, losses of the free acids ( $C_2$  to  $n-C_8$ ) were found to be much less (5–25% for the indicated acids). Also, the extent of loss was constant over a tenfold concentration of acids in ether, thus permitting quantitative comparisons of volatile acids in different samples.

In general, bright, burley, Maryland, and Turkish tobaccos gave qualitatively similar chromatograms. On a programmed DEGA-phosphoric acid column, major peaks corresponding to certain common fatty acids from  $C_1$  or  $C_2$  up to  $C_{16}$  were obtained. Since the higher fatty acids of tobacco had

been previously studied (19), work was limited to acids eluting up to and including pelargonic. Chromatograms of bright and Turkish tobaccos obtained on an isothermally operated DEGA column are shown in Figs. 1 and 2. Co-chromatography of the concentrates from all tobacco samples showed that the peaks from all tobacco had similar eluting patterns. On the basis of work reported below, the peaks were identified as indicated in the legend of Fig. 1. Isocaproic acid eluted as an inflection on  $\beta$ -methylvaleric acid, since the former was present in much smaller amounts. Also, it should be noted that small amounts of a substance eluting with peak 1 were obtained from some batches of solvent (ether) in blank runs. The quantitative contribution of this substance to the chromatograms was negligible.

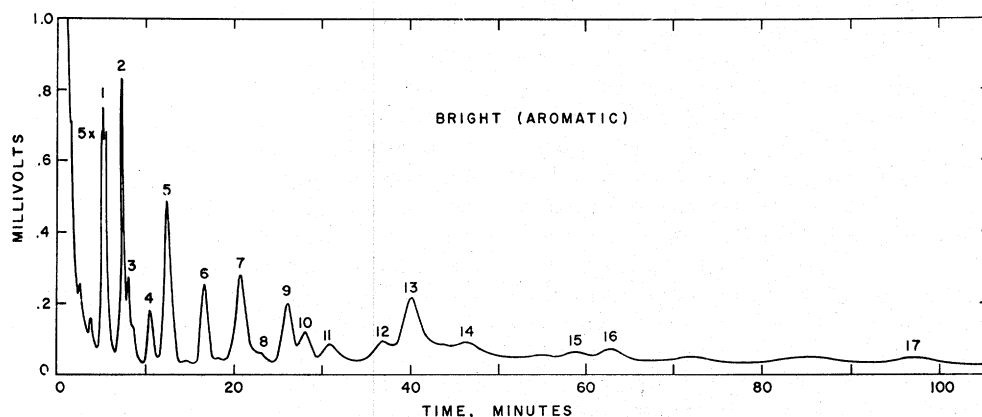


Fig. 1—Steam-volatile acids of bright tobacco (Aromatic A) separated on diethylene glycol adipate polyester-phosphoric acid column at 125°C (see Method for details). Peak identities: peak 1, formic and acetic; 2, propionic; 3, isobutyric; 4, *n*-butyric; 5, isovaleric; 6, *n*-valeric; 7,  $\beta$ -methylvaleric; 8, isocaproic; 9, *n*-caproic; 13, *n*-heptylic; 16, *n*-caprylic; 17, *n*-pelargonic; 10, 11, 12, 14, 15, unknown.

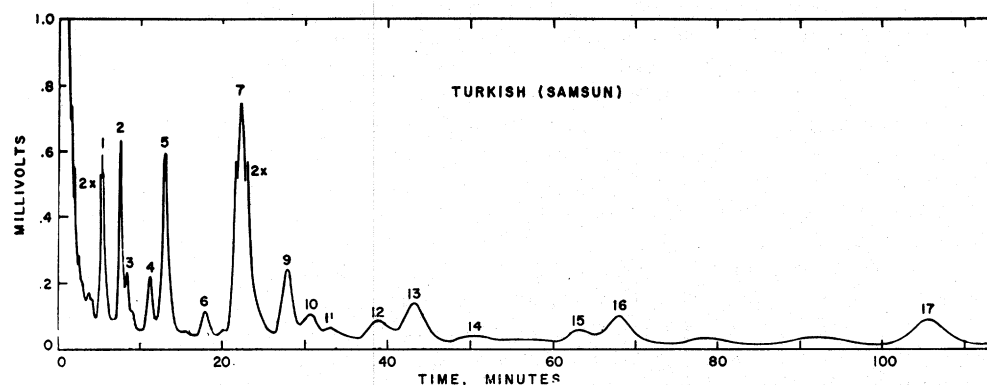


Fig. 2—Steam-volatile acids of Turkish (Samsun) tobacco. Chromatographic conditions and peak identities are given in Fig. 1.

The identifications of the peaks were based on comparisons of retention times of unknown and authentic compounds, by co-chromatography of knowns and unknowns on the two stationary phases, by paper chromatography of the entire volatile acid fraction or of collected peaks from gas chromatographic separations, and by infrared spectra of such collected peaks.

In some cases, conclusive evidence of identity by infrared spectra was difficult because of spectral similarities of certain aliphatic branched chain acids, e.g., isovaleric, isocaproic, and  $\beta$ -methylvaleric. Although the infrared spectrum of the peak corresponding to  $\beta$ -methylvaleric acid was identical to that of the authentic acid, it was not significantly different from that of authentic isocaproic acid. Differentiation between the two acids was demonstrated by co-chromatographing the unknown with each of the authentic acids independently. Authentic isocaproic acid eluted shortly after the unknown peak as a shoulder, but authentic  $\beta$ -methylvaleric acid eluted simultaneously with the unknown peak, increasing its height and maintaining its symmetry. Possibly other branched chain  $C_6$  isomers might also elute with the peak in question, and be likewise indistinguishable by infrared spectral examination. However, the presence of such isomers in tobacco or, for that matter, in any plant, has not been previously reported according to Karrer (20) but several workers have shown the presence of  $\beta$ -methylvaleric acid in tobacco leaves (4, 21). For these reasons, the unknown peak has been identified as  $\beta$ -methylvaleric acid.

According to a recent review (9), *n*-butyric, *n*-heptylic, *n*-caprylic, and *n*-pelargonic acids have not been previously reported in tobacco leaf.

The quantitative differences in burley, Maryland, Turkish (Samsun and Smyrna), and bright (three aromatic and two aroma-deficient grades) are given in Table 1, which includes values for ratios of equivalent peak areas (EPA), EPA per peak (for Aromatic A), and total EPA. The ratios were arbitrarily based on the pertinent EPA value for Aromatic A. The isothermally operated DEGA-phosphoric acid column with flame

ionization detection was used in obtaining these values. The table does not include data on three small inflections on peaks 3, 5, and 17 in certain samples; these inflections contributed insignificantly to the over-all evaluation of the samples.

Compared to the bright tobaccos, Turkish (Samsun) contained a much higher level of  $\beta$ -methylvaleric acid (greater than four times) and a lower level of combined acetic and formic acids (less than one-half). The proportion of isovaleric to *n*-valeric acid in Turkish (Samsun) was higher compared to the bright tobaccos. Turkish (Smyrna) was generally similar to Turkish (Samsun) in the pattern of distribution of acids but the former contained much lower levels. Burley and Maryland contained lower levels of all identified acids than Turkish (Samsun) and bright.

Among the samples of bright tobaccos differing in aroma, the aromatic tobaccos generally showed higher total EPA and larger amounts of the lower acids than the aroma-deficient tobaccos, although at least one exception was noted (*n*-butyric). The ratios of iso-acids to parent *n*-acids for  $C_4$  and  $C_5$  were higher in the aromatic tobaccos, although the difference between Aromatic B and Aroma-deficient B was not great for the  $C_5$  isomers. In general, the quantitative patterns of acids in the various samples paralleled the previously reported findings on the volatile neutral substances in these tobaccos.

The levels of the identified acids in the tobaccos were determined from area-concentration curves of authentic acids, and representative findings are shown in Table 2. These data illustrate the magnitude of values obtained and the differences in proportions of acids in Turkish and bright. Differences between the relative amounts of total acids and the relative total EPA values (Table 1) for the bright and Turkish are due to the effect of pooling EPA values of peaks with different detector responses. In comparing samples largely dissimilar in the proportions of acids, e.g., Aromatic A and Turkish, such differences are maximal; comparisons of total levels of acids are probably more reliable than total EPA in these in-

Table 1. Quantitative differences in certain steam-volatile acids of various tobacco types and grades

Ratios of Equivalent Peak Areas (EPA) for Indicated Peak Numbers <sup>a</sup>										
Peak	No. of Acids	Bright					Burley	Maryland	Turkish	
		Aromatic			Aroma-Deficient					
		A	B	C	A	B				
1	C <sub>1</sub> -C <sub>2</sub>	1.0	0.95	0.99	0.77	0.82	0.05	0.10	0.09	0.37
2	C <sub>3</sub>	1.0	0.91	1.17	0.71	0.69	0.24	0.38	0.41	0.73
3	<i>i</i> -C <sub>4</sub>	1.0	0.97	0.63	0.20	0.20	0.08	0.04	0.32	0.48
4	<i>n</i> -C <sub>4</sub>	1.0	0.65	0.69	0.77	0.69	0.08	0.12	0.08	1.18
5	<i>i</i> -C <sub>5</sub>	1.0	0.55	1.26	0.34	0.39	0.08	0.18	0.41	0.94
6	<i>n</i> -C <sub>5</sub>	1.0	0.74	0.90	0.63	0.61	0.04	0.07	0.08	0.41
7	BMV	1.0	0.52	0.88	0.41	0.41	0.06	0.22	0.98	4.84
8	<i>i</i> -C <sub>6</sub>	1.0	0.84	1.00	0.71	1.01	—	0.16	—	—
9	<i>n</i> -C <sub>6</sub>	1.0	0.65	0.71	0.56	0.80	0.14	0.33	0.15	1.23
10	U	1.0	0.62	0.72	0.33	0.55	0.08	0.09	0.19	1.24
11	U	1.0	0.96	1.04	0.90	1.21	0.13	0.54	0.17	0.82
12	U	1.0	0.45	0.69	0.91	0.76	0.14	0.37	0.18	1.26
13	<i>n</i> -C <sub>7</sub>	1.0	0.46	0.59	0.59	0.42	0.34	0.42	0.15	0.69
14	U	1.0	0.58	0.54	0.45	0.32	0.11	0.69	—	0.44
15	U	1.0	1.26	1.21	1.27	0.99	0.73	0.79	0.33	1.64
16	<i>n</i> -C <sub>8</sub>	1.0	0.66	0.75	1.28	0.75	0.46	0.53	0.28	2.42
17	<i>n</i> -C <sub>9</sub>	1.0	2.53	1.14	3.91	1.80	0.52	0.60	0.33	2.15
Total EPA <sup>b</sup>		22,700	19,000	21,600	17,400	16,200	2,930	5,100	5,100	22,100

<sup>a</sup> Identities of peaks are given beside peak numbers. BMV =  $\beta$ -methylvaleric acid, U = unknown. EPA values for Aromatic A for peaks 1-17 were as follows: 10,340, 1694, 281, 402, 1854, 825, 1551, 77, 974, 402, 350, 484, 1672, 517, 303, 429, 611. The indicated values were those obtained and are not corrected for losses due to volatility (see text).

<sup>b</sup> Rounded off to three significant figures.

**Table 2. Levels of volatile acids in various tobaccos ( $\mu\text{g}$  acid per 100 g tobacco<sup>a</sup>)**

Acid	Bright		Turkish (Samsun)
	Aromatic A	Aroma- Deficient A	
Formic-acetic	2302	1773	852
Propionic	209	148	153
Isobutyric	26	5	12
n-Butyric	38	29	45
Isovaleric	181	62	170
n-Valeric	70	44	29
$\beta$ -Methylvaleric	211	87	1021
n-Caproic	67	38	82
n-Heptylic	119	70	82
n-Caprylic	34	44	82
n-Pelargonic	46	180	99
Unknowns <sup>b</sup>	39	105	149
Total	3442	2585	2776

<sup>a</sup> Moisture-free basis.<sup>b</sup> Calculated as n-caproic acid.

stances. However, in comparing samples which are very similar, e.g., the above aromatic grades, good correlation between total EPA and total levels of acids is observed and total EPA can be used in this way. Of course, comparison of EPA values for a given peak in the different samples is completely valid as an index of amount of acid present, provided the peaks from all samples are kept within the range of linearity of detector response and concentration, as was done here.

#### Acknowledgments

Grateful appreciation is expressed to Dr. D. Burdick, Mrs. W. R. Bilinsky, and Mr. R. L. Chrzanowski for their assistance in this investigation.

#### REFERENCES

- (1) Burdick, D., Schmeltz, I., Miller, R. L., and Stedman, R. L., *Tobacco Sci.*, **7**, 97 (1963).
- (2) Burdick, D., and Stedman, R. L., *ibid.*, **7**, 113 (1963).
- (3) Onishi, I., and Yamamoto, K., *Bull. Agr. Chem. Soc. Japan*, **21**, 90 (1957).
- (4) Onishi, I., and Yamasaki, K., *ibid.*, **20**, 68 (1956).
- (5) *Ibid.*, **21**, 86 (1957).
- (6) *Ibid.*, **19**, 137 (1955).
- (7) Buyske, D. A., Wilder, P., Jr., and Hobbs, M. E., *Anal. Chem.*, **29**, 105 (1957).
- (8) Izawa, M., and Kobashi, Y., *Bull. Agr. Chem. Soc. Japan*, **22**, 47 (1958).
- (9) Johnstone, R. A. W., and Plimmer, J. R., *Chem. Rev.*, **59**, 885 (1959).
- (10) Tobacco Mfgs. Standing Committee Research Papers, No. 3, "The Constituents of Tobacco Smoke: An Annotated Bibliography," edited by H. R. Bentley and E. G. N. Berry, London, 1959.
- (11) Schmeltz, I., Miller, R. L., and Stedman, R. L., *J. Gas Chromatography*, in press.
- (12) Stedman, R. L., and Miller, R. L., *J. Chromatography*, **11**, 409 (1963).
- (13) Hammarberg, G., and Wickberg, B., *Acta Chem. Scand.*, **14**, 882 (1960).
- (14) Isherwood, F. A., and Hanes, C. S., *Biochem. J.*, **55**, 824 (1953).
- (15) Reid, R. L., and Lederer, M., *ibid.*, **50**, 60 (1951).
- (16) Schwartzman, G., *This Journal*, **43**, 428 (1960).
- (17) Bacot, A. M., U.S. Dept. of Agriculture, Tech. Bull. No. 1225 (1960).
- (18) Phillips, M., and Bacot, A. M., U.S. Department of Agriculture, Tech. Bull. No. 1186 (1958).
- (19) Swain, A. P., and Stedman, R. L., *This Journal*, **45**, 536 (1962).
- (20) Karrer, W., "Konstitution und Vorkommen der organischen Pflanzenstoffe," Birkhäuser Verlag, Basel und Stuttgart, 1958.
- (21) Sabetay, S., and Panouse, J., *Compt. rend.*, **225**, 887 (1947).